

DEVELOPMENT AND CLINICAL VALIDATION OF AN INTEGRATED RAPID POINT OF CARE WORKFLOW FOR DETECTION OF *bla*_{CTX-M-15} AND *aac* (6')-Ib-CR ANTIMICROBIAL RESISTANCE GENES IN URINARY TRACT INFECTION URINE AT RESOURCE LIMITED HOSPITALS AND LABORATORIES.

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ABSTRACT

Background: The Rapid detection of antimicrobial resistance (AMR) is essential for periodically diagnosing for antimicrobial analysis, yet the conventional antimicrobial susceptibility testing (AST) method still requires a minimum of 24–72 hours. This study developed and validate an integrated point-of-care real-time PCR workflow for rapid detection of the *bla*_{CTX-M-15} and *aac* (6')-Ib-cr resistance genes extracted from urine specimens.

Methods: A total of 150 urine samples from adults with suspected urinary tract infections were analyzed using HiPurA Genomic DNA extraction Kits followed by real-time PCR. This diagnostic is a simple molecular workflow which is rapid, simple, and cost effectiveness and can be used in low resource laboratories in the detection of antimicrobial resistance. It has been was evaluated against the phenotypic AST and other already existed molecular methods,

Results: The complete sample to result molecular workflow was completed within approximately 60 minutes. The workflow was demonstrated at high diagnostic performance for *bla*_{CTX-M-15} with the diagnostic threshold of 94.74% sensitivity and a specificity of 90.91% followed by the other resistance gene which *aac* (6')-Ib-cr which was having a diagnostic performance of sensitivity 92.63% and specificity of 92.73% with the reference to the Antimicrobial Susceptibility Testing method.

Conclusions: This study involves basically present a rapid molecular and PCR workflow which is directly extracted from urine sample targeting *bla*_{CTX-M-15} and *aac* (6')-Ib-cr within a time space of 60minutes

KEYWORDS: Antimicrobial resistance, Point-of-care diagnostics, Real-time PCR; Urinary tract infection; *bla*_{CTX-M-15}, *aac* (6')-Ib-cr, molecular diagnostics, Antimicrobial stewardship.

INTRODUCTION

The Global Burden of AMR and Diagnostic Gaps

Resistance against antimicrobials (AMR) stands now as a major global public health issue causing roughly 1.27 million deaths annually and greatly raising sickness levels, medical expenses, and therapy failure rates globally [1]. The urinary tract infections (UTIs) are mainly linked to bacterial causes which includes *Escherichia coli* and *Klebsiella pneumoniae*, represent among the frequentist infection kinds found within both community and hospital settings [2]. The Rising numbers of ESBL multidrug-resistant urinary pathogens have provided an empirical diagnosis of infections hard and demand quick spotting of resistance genes [3].

The Standard microbial diagnosis basically depends on the growing of the bacteria on a culture media then performing antimicrobial susceptibility testing (AST), staying the gold benchmark for finding phenotypic drug sensitivity levels. While these approaches offer full details regarding species ID and resistance patterns, they usually need twenty-four to seventy-two hours until medical decisions can act upon data [4]. Such waiting periods frequently force doctors to start wide-range antibiotic treatment early possibly leading to wrong medicine choices longer stays in hospitals, worse patient health results, and creating more drug-resistant bugs [5].

New progress in molecular diagnostics has greatly enhanced how quickly we find AMR. Commercial real-time PCR tools and combined sample-to-answer systems, such as cartridge-based molecular tests, can fast spot chosen resistance genes straight from patient samples using high analytical sensitivity and specificity [6]. These technologies show great clinical worth by cutting diagnostic turnaround time and helping make therapy choices sooner. Yet, their broad use stays restricted in many medical places due to expensive instruments, owned-up consumables, need for central lab setup, and low flexibility for test changes [7]. Also, many sold tests work best in rich labs and might be hard to use often in scattered or poor health care spots.

The most prevalent resistance gene that causes cephalosporin-resistant, third-generation urinary microbes is *bla*_{CTX-M-15}, the most common genes associated with aminoglycoside resistance and to a lesser extent, fluoroquinolone resistance, are *aac* (6')-Ib-cr. These genes are located on a mobile genetic component which are capable of horizontal transfer between related Enterobacterales bacteria [8].

The goal of this research is to create and validate an integrated point of care real-time polymerase chain (PCR) testing workflow for a quick and reliable identification of both the *bla*_{CTX-M-15} and *aac* (6')-Ib-cr genes directly from urine specimens collected from individuals suspected with urinary tract infection (UTI). The planned testing workflow utilizes rapid magnetic bead DNA extraction, optimized PCR amplification, as well as a streamlined process for interpreting the results to generate clinically actionable results within approximately one hour. While the new testing technology does not represent a new PCR methodology, it does evaluate an optimized molecular diagnostic workflow that enhances the practicality of rapid detection of antibiotic resistance while maintaining high diagnostic accuracy when compared to conventional phenotypic antimicrobial susceptibility testing methods.

Research Gap

Currently studies have done by extracting AMR genes such as *bla*_{CTX-M-15}, *bla*_{CTX-M-9}, *bla*_{TEM}, *bla*_{SHV-12}, *bla*_{CMY-2}, *bla*_{OXA-48}, *bla*_{NDM-1} and *bla*_{IMP-1} directly urine samples within a time of 3 hours [9]. But this research basically composes *bla*_{CTX-M-15} and *aac* (6')-Ib-cr. AMR genes which was extracted from urine basically using the HiGenoMB MB573 DNA test kit. After the integrated work from DNA extraction and PCR analysis were conducted in a molecular workflow for this AMR detection was able to liberate or give out result within a frame of one hour.

Study Objectives and Innovation

This study presents the developed and analytically validated point-of-care molecular diagnostic workflow which combine a rapid magnetic bead-based DNA extraction, optimized real-time PCR amplification and result interpretation for detection of *bla*_{CTX-M-15} and *aac*(6')-Ib-cr specifically from a urine specimens, which was extracted directly from midstream urine samples. These targets were selected due to their high prevalence in Enterobacteriaceae and their recurrent co-residency on itinerant genetic elements giving resistance to beta-lactams, aminoglycosides, and fluoroquinolones.

MATERIALS AND METHODS

Study Design and Ethical Approval

This experimental study was conducted at Parul Sevashram Hospital (Vadodara-Gujarat India) to assess a new point-of-care (POC) molecular diagnostic platform for antimicrobial resistance (AMR). The study protocols were approved by the Institutional Ethics Committee of the Parul Institute of Medical Sciences and Research Approval No: PUIECHR/PIMSR/00/081734/8126.

A purposive sample of 150 adult patients with the age group greater than and equal to 18years presenting with clinical symptoms of a urinary tract infection (UTI) from both outpatient and inpatient departments was included. Patients were excluded if they had received some antibiotic therapy for more than 72 hours earlier to collection and patients which possessed indwelling urinary catheters for more than 7 days had some known structural urinary tract abnormalities or were suspected of some viral and fungal infections.

Sample Collection and DNA Extraction

A Clean-catch of a midstream urine sample (10–20 mL) were collected in sterile polypropylene containers and processed within 30 minutes at 4°C. Samples were gently inverted and a 1 mL aliquot was transferred to a microcentrifuge tube for other residual extraction process.

Genomic DNA was isolated directly from the midstream urine using the HiPurA Urine DNA Purification Kit (HiGenoMB MB573) optimized for a 20-minute workflow. 200 µL of Lysis Solution (C1) and 20 µL of Proteinase K were added to the 1 mL of urine sample in an aliquot tube and vortexed process was applied to mix the solutions homogeneously and incubated at room temperature for 5 minutes. The lysate was then transferred to a HiElute Miniprep spin column and centrifuged for (8000 x g for 1 min) after the remaining residua was washed sequentially with 500 µL of Wash Buffer and the DNA was eluted in 50 µL of pre-warmed Elution Buffer (10 mM Tris-Cl with pH 8.5) and quantified by a spectrophotometry of A260/A280 with the reference range of 1.0–1.9) prior to analysis.

The design of a Prime and Real-Time PCR Reaction Framework

The *bla*_{CTX-M-15} and *aac* (6')-Ib- six primer pairs were selected for the specific amplification of clinically relevant antimicrobial resistance genes. Primer design was designed using NCBI Primer-BLAST to ensure that the target specificity and to minimize non-specific amplification. The primer sequences were subsequently verified by BLASTN against the NCBI nucleotide database to confirm the specificity for the intended targeted genes. The primers were designed to produce short amplicons which are suitable for real-time PCR amplification and point-of-care molecular diagnostics.

Table 1: Primer Sequences for AMR Target Genes

Target Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
<i>bla</i> _{CTX-M-15}	ATGTGCAGYACCAGTAARGT	TTACAAACCGTYGGTGACGA

<i>aac (6')-Ib-cr</i>	TTGCGATGCTCTATGAGTGGCTA	CTCGAATGCCTGGCGTGTTT
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Real-Time PCR Amplification and Detection Protocol

Following DNA extraction and quality assessment, all DNA samples meeting the predefined quality criteria (A260/A280 ratio of 1.62–1.89) underwent real-time polymerase chain reaction (RT-PCR) targeting the antimicrobial resistance genes *bla*_{CTX-M-15} and *aac (6')-Ib-cr*. This amplification protocol was constructed to enable rapid, sensitive, and specific identification of clinically significant resistance markers directly within urine-sourced bacterial genetic material. The assay employed gene- forward and reverse primers specific sequences created via the NCBI Primer-BLAST system. Specificity verification used in silico BLAST checks to stop non-specific amplification and cross-reaction with other bacterial genomes. Short amplicons roughly 100 to 150 bp chosen to boost amplification speed, cut reaction duration, and better assay results on portable real-time PCR devices fit for point-of-care tests.

PCR Reaction Mixture

Each PCR reaction was prepared in a final volume of 20 µL consisting of the following components:

Table 2: the PCR reaction mixtures and their appropriate volume

PCR Component	Volume (µL)
2× TaqMan Universal Master Mix	10.0
Forward Primer (250 nM final concentration)	0.5
Reverse Primer (250 nM final concentration)	0.5
TaqMan Hydrolysis Probe (125 nM final concentration)	0.5
Template DNA	2.0
Nuclease-Free Water	6.5
Total Reaction Volume	20.0

The entire reaction mixture preparation was done in a PCR clean area by employing sterile aerosol resistant filter tips to minimize contamination. Different workstations were used for preparation of reagents, addition of DNA template, and post amplification analyses as per the standard lab practice in molecular biology.

Thermal Cycling Conditions

The amplification was carried out on a portable real-time PCR thermocycler which is a device used in molecular diagnostics. The protocol for cycling was an initial enzyme activation step at 95°C for 10 min, then 40 amplification cycles that included denaturation at 95°C for 15 sec and annealing and extension at 60°C for 60 sec combined. These cycling conditions were designed and tested to give the best amplification efficiency with the greatest analytical specificity.

Fluorescence Detection and Data Acquisition

During each PCR cycle, fluorescence signals produced due to amplification were continuously observed. The instrument software automatically determined the cycle threshold (Ct) value, which is the amplification cycle number where fluorescence crossed the set threshold level. If a sample's Ct value was less than 35, it was considered that the sample contained the respective antimicrobial resistance gene. In contrast, samples with Ct values above 40 or samples without any detectable amplification were categorized as negative. If samples resulted in Ct values within the range of 35 to 40, they were subject to repeat amplification before a final decision was made.

Quality Control Measures

A positive control consisting of Previously characterized DNA carrying the targeted resistance gene was included to check the functionality of the assay whereas no-template control (NTC) containing nuclease-free water was used to check for reagent contamination. The amplification runs were only regarded as correct when the positive control exhibited a clear and expected amplification curve and the NTC continuously stayed negative during the entire run.

DNA concentration and purity were measured spectrophotometrically at ratios of A260/A280, and only DNAs with satisfactory purities were taken forward to be checked further with molecular techniques.

Diagnostic Interpretation

Sizes of the target PCR products for the *bla*_{CTX-M-15} and *aac* (6')-Ib-cr genes were then compared to the results of standard antimicrobial susceptibility testing (AST) done based on the Clinical and Laboratory Standards Institute (CLSI) guidelines. Diagnostic performance was first assessed by calculating the numbers of true-positive, true-negative, false-positive, and false-negative results. Then, from those numbers, the parameters of sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and overall diagnostic accuracy were obtained.

DNA extraction was combined with careful spectrophotometric quality assessment, primer design optimization, and real-time PCR amplification to accomplish the molecular diagnostic procedure in about 60 min time, thus fulfilling the desired function of the assay as a point-of-care diagnostic platform for antimicrobial resistance detection in urinary pathogens (*bla*_{CTX-M-15} and *aac*(6')-Ib-cr).

RESULT

This finding analytically validated and integrated a point-of-care molecular diagnostic workflow that combined rapid magnetic bead-based DNA extraction to optimized real-time PCR amplification and streamlined the result interpretation for detecting *bla*_{CTX-M-15} and *aac* (6')-Ib-cr in urinary tract infection samples. A total number of 150 patients were recruited, and their urine samples underwent microbiological analysis, DNA extraction, DNA quality assessment, real-time PCR amplification and were compared with reference of antimicrobial susceptibility testing (AST) method.

The findings in this paper will be shown step-by-step to explain the entire diagnostic workflow. First, the demographic and clinical characteristics of the study population are presented. Next, the DNA quality and primer performance are assessed. Then, the molecular detection of the target antimicrobial resistance genes is presented along with their diagnostic performance compared to the reference AST method [10,11,12]. Lastly, the workflow efficiency, turnaround time, and the comparative performance of the developed assay with conventional diagnostic methods are reported to show its potential clinical applicability for point-of-care antimicrobial resistance detection.

Statistical Analysis

IBM SPSS Statistics was used to analyses the statistical outcome. The Descriptive statistics were aimed at providing a summary of the demographic attributes and the distribution of the pathogens, DNA purity (A260/A280 ratio), and the real-time PCR results. Continuous variables were shown as either mean, standard deviation (SD) or median (interquartile range [IQR]) where it is suitable, whereas categorical variables are displayed by means of their frequency and percentages.

Demographic Characteristics of the Study Population

A total number of 150 adult patients with clinically suspected urinary tract infections (UTIs) were enrolled in the study. The age of this participant ranges from 18 to 75 years with the largest proportion belonging to the 31–40-year age group representing (24.0%). The number of females constituted 61.3% (92/150) of the study population while males reported for 38.7% (58/150). *Escherichia coli* was the most frequently isolated uropathogens which represented (55.3%) followed by *Klebsiella pneumoniae* which also represent (19.3%). Antimicrobial susceptibility test shown several numbers of 95 representing (63.3%) resistant isolates and 55 susceptible isolates representing (36.7%). The Molecular examination detected the *bla*_{CTX-M-15} resistance gene in 68 (45.3%) isolates and the *aac* (6')-Ib-cr gene in 61 (40.7%) isolates. Co-occurrence of both resistance genes was observed in 42 (28.0%) isolates.

Table 3: Demographic Characteristics of the Study Population

Variable	Category	Number =150	Percentage (%)
Age Group (years)	18–30	28	18.7
	31–40	36	24.0
	41–50	34	22.7
	51–60	27	18.0
	>60	25	16.6
Sex	Male	58	38.7
	Female	92	61.3
Predominant Pathogen Isolated	<i>Escherichia coli</i>	83	55.3
	<i>Klebsiella pneumoniae</i>	29	19.3
	<i>Enterococcus faecalis</i>	16	10.7
	<i>Pseudomonas aeruginosa</i>	12	8.0
	<i>Proteus mirabilis</i>	6	4.0

	Other Gram-negative bacteria	4	2.7
Phenotypic Resistance (AST)	Resistant isolates	95	63.3
	Susceptible isolates	55	36.7
bla _{CTX-M-15} Gene (PCR)	Positive	68	63.3
	Negative	55	36.7
aac (6')-Ib-cr Gene (PCR)	Positive	62	61.3
	Negative	58	38.7
Co-harboring Both Genes	Positive	42	28.0
	Negative	108	72.0

DNA Purity Assessment

The quality of extracted DNA is a perilous determinant for PCR performance. In this study the DNA purity was assessed using the A260/A280 absorbance ratio. All samples demonstrated a values varying from 1.62 to 1.89 which falls within the acceptable range for the DNA purity assessment.

The results confirm that the DNA extraction protocols were both efficient and dependable resulting with high-quality of nucleic acid which is suitable for downstream amplification.

Table 4: showing the DNA purity assessment

Parameter	Result
Total urine samples processed	150
DNA successfully extracted	150
DNA purity assessment method	(Spectrophotometry) (A260/A280)
Mean A260/A280 ratio	1.76 ± 0.08
Median A260/A280 ratio	1.77
Range	1.62–1.89
Samples within acceptable purity range (1.6–2.0)	150 (100%)

Table 5: Showing the Diagnostic Performance of aac (6')-Ib-cr Assay

Diagnostic Outcome	Number of Samples
True Positive (TP)	88
True Negative (TN)	51
False Positive (FP)	4
False Negative (FN)	7
Total Samples	150

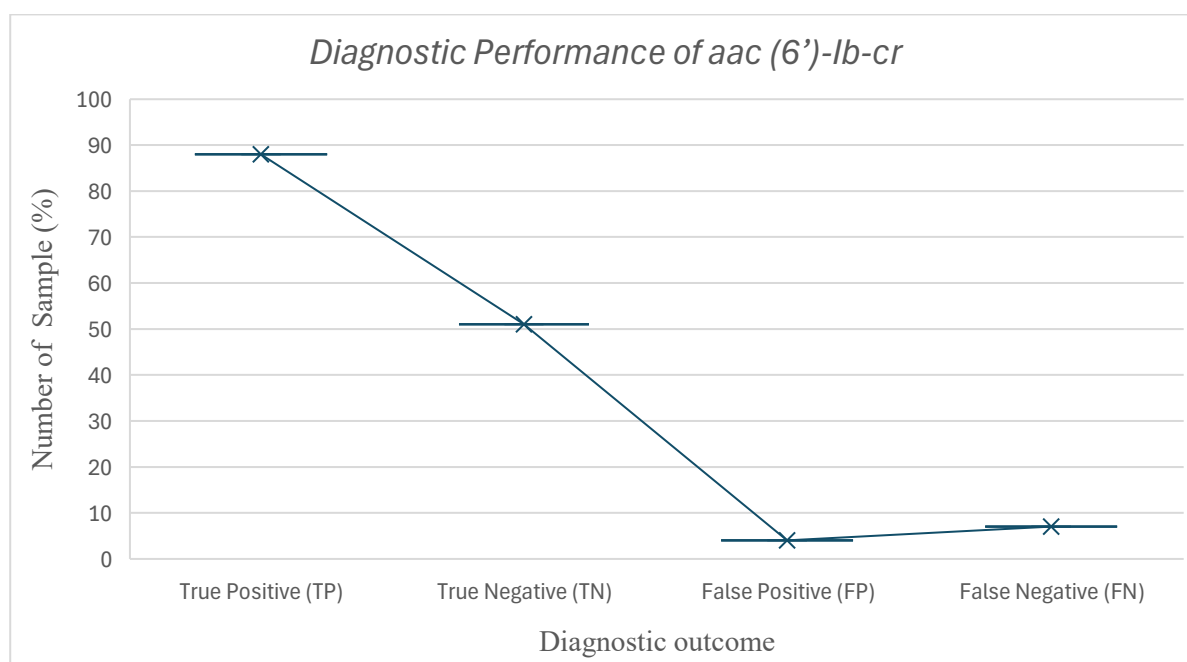


Figure 1: Diagnostic Performance of aac (6')-Ib-cr

Diagnostic Performance of the aac (6')-Ib-cr workflow

The real-time PCR assay that targets the aac (6')-Ib-cr resistance gene was tested on 150 urine samples from patients. These samples were compared to the antimicrobial susceptibility testing method as a reference. Out of the 150 samples 88 were found to be positives, which means they correctly showed that the samples had the aac (6')-Ib-cr gene that makes bacteria resistant to antibiotics. 51 Samples were negatives which means they correctly showed that the samples did not have any of this gene.

The workflow assay was very good at finding the aac (6')-Ib-cr gene in this bacteria gene. It found it in 92.63% of the cases. It was also very good at ruling out the gene in bacteria that were not resistant. It did this correctly in 92.73% of the cases. If the test said a sample was positive it was usually 95.65% of the time. If it said a sample was negative it was mostly right too. 87.93% Of the time.

Overall, the real-time PCR assay was 92.67% which is very close to the standard test. This means it is a way to test for the aac quickly and reliably (6')-Ib-cr resistance gene in urine samples. The test could be used to help doctors choose the antibiotics for patients with urinary tract infections that are resistant to certain antibiotics.

Table 6: Diagnostic Performance Statistics of the aac (6')-Ib-cr gene

Parameter	Value
Sensitivity	92.63%
Specificity	92.73%
Positive Predictive Value (PPV)	95.65%
Negative Predictive Value (NPV)	87.93%
Overall Accuracy	92.67%

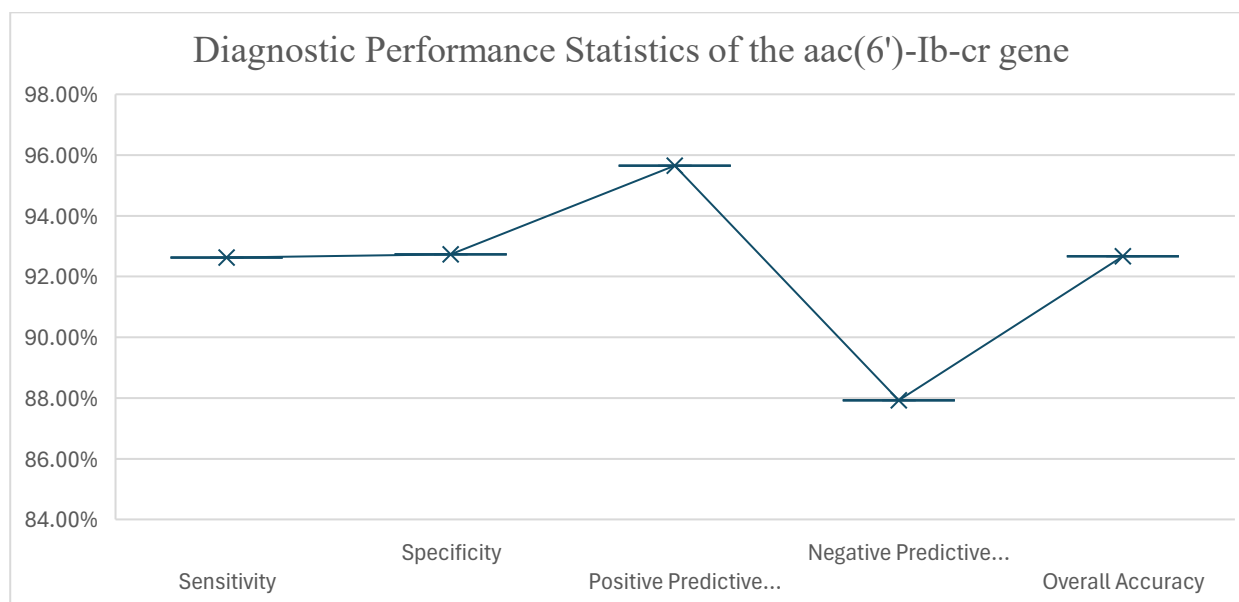


Figure 2: performance statistics of the aac (6')-Ib-cr gene

STATISTICAL PERFORMANCE OF THE AAC (6')-IB-CR ASSAY

The optimized of this workflow has targeted the aac (6')-Ib-cr and demonstrated a high diagnostic performance when it is compared with the reference AST method. The Sensitivity was 92.63% and it was having a specificity threshold of 92.73%. The positive predictive value was 95.65% and the negative predictive value was 87.93% and the overall diagnostic accuracy was 92.67%

A highly significant association was analyzed between PCR and AST results using the (Pearson's $\chi^2 = 107.02$, $df = 1$, $P < 0.001$). Agreement between the two methods was almost perfect. (Cohen's $\kappa = 0.844$). McNemar's test demonstrated no statistically significant disagreement between paired results ($\chi^2 = 0.36$, $P = 0.546$). ROC curve analysis yielded an AUC of 0.927.

Table 7: Showing diagnostic Performance of bla_{CTX-M-15} Assay

Diagnostic Outcome	Number of Samples
True Positive (TP)	90
True Negative (TN)	50
False Positive (FP)	5
False Negative (FN)	5
Total Samples	150

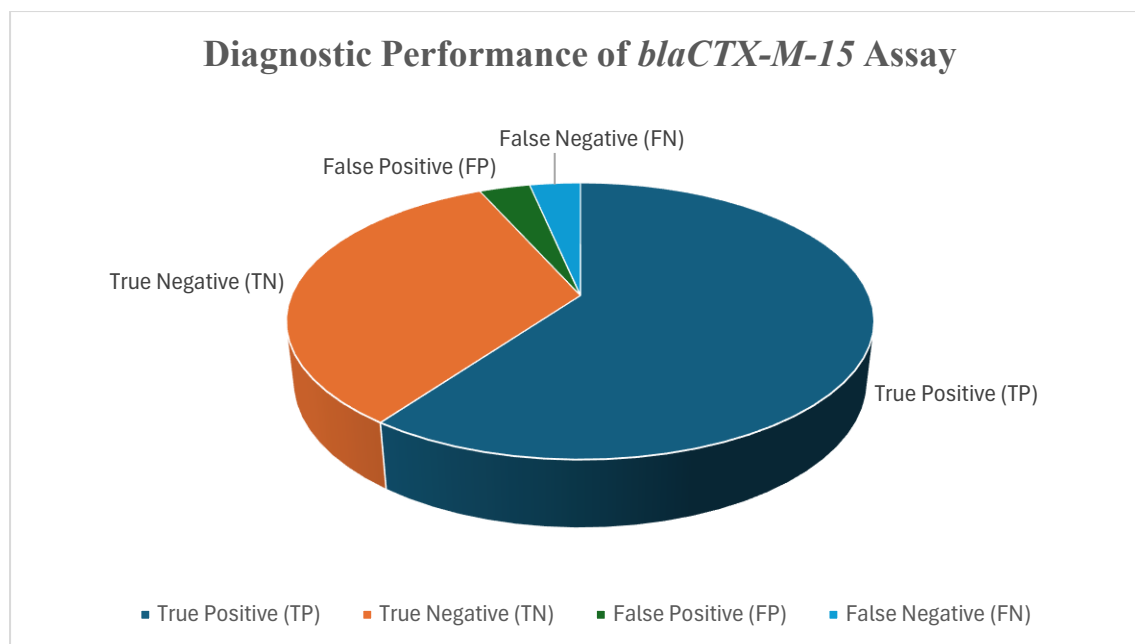


*Figure 3: The diagnostic performance of the *bla*_{CTX-M-15} assay*

Diagnostic Performance of the *bla*_{CTX-M-15} workflow Assay

To evaluate how well the new real-time PCR assay detects the *bla*_{CTX-M-15} resistance gene. We tested 150 clinical urine samples and compared the results directly against standard antimicrobial susceptibility testing (AST). Since every single sample went through both the molecular test and traditional phenotypic AST, we were able to get a complete picture of the workflow performance.

Out of the 150 specimens the PCR method was correctly shown with 90 true positives and 50 true negatives. When it was measured against the AST reference standard, we only saw five false positives and five false negatives. This breakdown evaluates the sensitivity of 94.74% which means the has successfully detect most resistant isolates carrying the *bla*_{CTX-M-15} marker. The Specificity was also 90.91% which highlights how reliable the test is and when it comes to properly identifying susceptible strains that lack the target gene.

The positive and negative predictive values mirrored these metrics exactly at 94.74% and 90.91%, respectively. The overall workflow achieved a diagnostic accuracy of 93.33%. This points to a remarkably strong agreement between the newer molecular approach and the traditional AST method.

What this essentially tells us is that targeting the *bla*_{CTX-M-15} gene with this optimized PCR workflow is a fast and dependable way to spot ESBL-associated resistance straight from urine. Given the high accuracy observed in the study the approach has a real potential to be used as a point-of-care diagnostic tool detecting ESBL-producing uropathogens earlier.

Table 8: Diagnostic Performance Statistics of the *bla*_{CTX-M-15} Assay

Diagnostic Parameter	Value
Sensitivity	94.74%
Specificity	90.91%
Positive Predictive Value (PPV)	94.74%
Negative Predictive Value (NPV)	90.91%
Overall Accuracy	93.33%
False Positive Rate	9.09%
False Negative Rate	5.26%

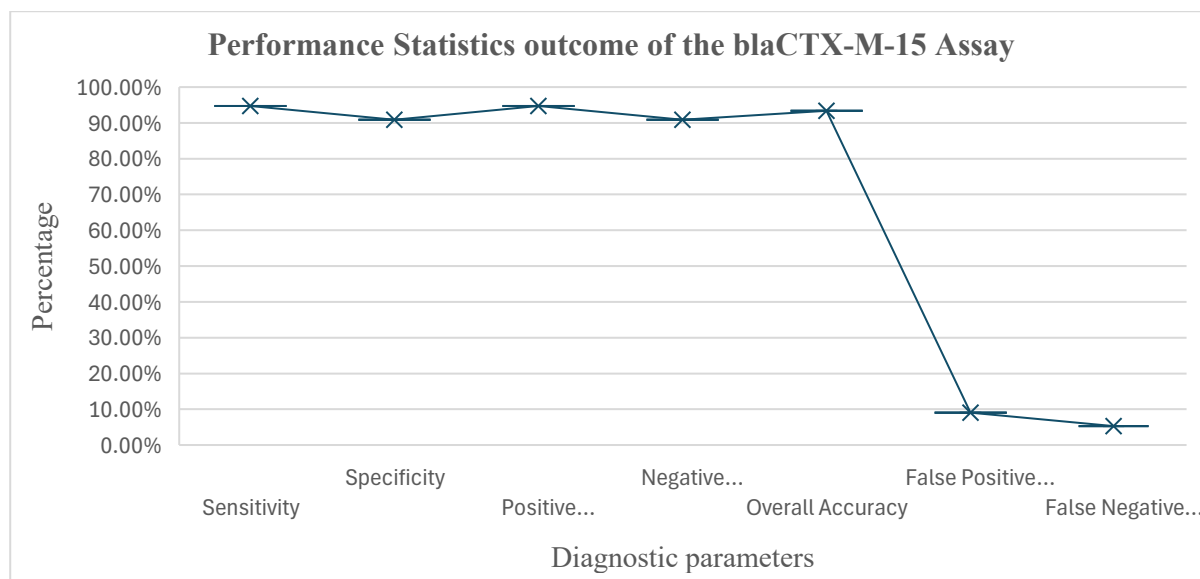


Figure 4: The performance statistics outcome of the bla_{CTX-M-15} assay

STATISTICAL PERFORMANCE OF THE BLA_{CTX-M-15} ASSAY

The optimized real-time PCR assay targeting bla_{CTX-M-15} demonstrated excellent diagnostic performance compared with the reference phenotypic AST method. The workflow has achieved a sensitivity of 94.74% and a specificity of 90.91% with a positive predictive value of 94.74% and a negative predictive value of 90.91% and an overall diagnostic accuracy of 93.33%

A highly significant association was observed between PCR and AST results with the (Pearson's $\chi^2 = 110.03$, df = 1, $P < 0.001$). Agreement between the two methods was almost perfect (Cohen's $\kappa = 0.856$). McNemar's test showed no statistically significant disagreement between paired results which was ($\chi^2 = 0.10$, $P = 0.752$) indicating the excellent concordance between this molecular workflow and the reference method. ROC analysis demonstrated performance with an area under the curve (AUC) of 0.928.

Workflow Efficiency and Turnaround Time

After successful DNA extraction, DNA quality assessment, primer validation, and real-time PCR amplification, the operational efficiency of the developed and analytically validated an integrated point-of-care molecular diagnostic workflow was evaluated by comparing its diagnostic workflow with conventional laboratory methods.

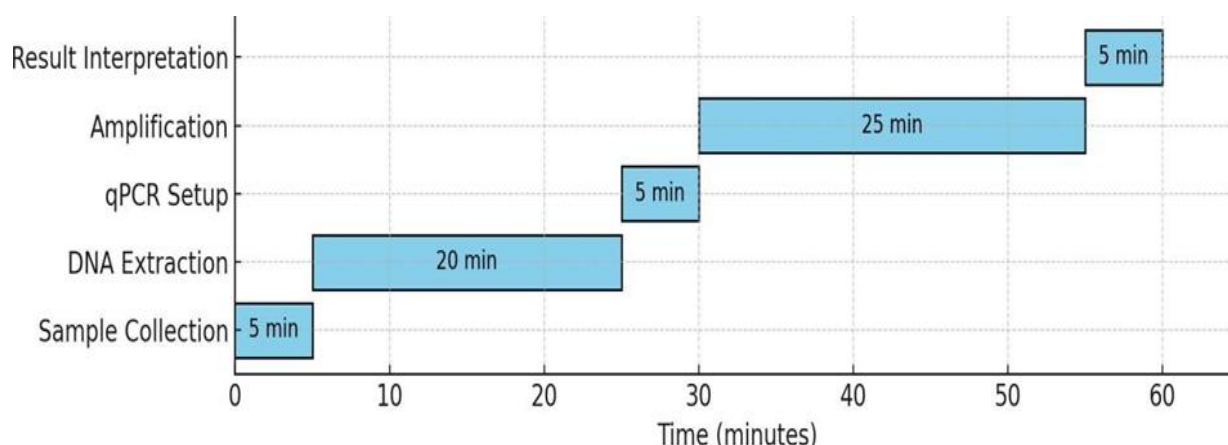


Figure5: Timeline of the Integrated Point-of-Care Molecular Diagnostic Workflow for Rapid Detection of bla_{CTX-M-15} and aac (6')-Ib-cr. The workflow illustrates the sequential steps from urine sample collection through rapid DNA extraction, DNA quality assessment, real-time PCR amplification, fluorescence detection, and result interpretation, with a total turnaround time of approximately 60 minutes

The rapid diagnostic method for the bla_{CTX-M-15} and aac (6')-Ib-cr offers a several advantages over the traditional culture-based AST methods. While the AST method remains the golden standard for antimicrobial susceptibility test it is also time-consuming, labor- demanding and dependent on specialized laboratory infrastructures. This integrated point-of-care assay provides a rapid, accurate and culture-independent detection for the resistance genes. The ability to generate results within a single clinical visit considerably enhances its clinical utility which particularly in emergency and resource-limited hospitals.

To assess the possible clinical value of the created test, its ability to work well was checked against normal lab ways usually used for finding drug resistance. As seen in Table 4 the made quick real-time PCR test looking at the *bla*_{CTX-M-15} and *aac* (6')-Ib-cr genes gave answers inside about one hour, showing a big drop-in time needed for diagnosis when compared to normal AST (about 24 hours) and growing cultures then doing AST (24 to 48 hours).

DISCUSSION

This research was molecular based workflow where midstream urine sample was directly extracted using the HiPurA Urine DNA Purification Kit (HiGenoMB MB573) and a primer was design using Silco to target the specific antimicrobial resistance i.e. *bla*_{CTX-M-15} and *aac*(6')-Ib-cr. The workflow combined rapid DNA extraction, spectrophotometric DNA-quality assessment, and real-time PCR amplification to generate molecular resistance information which has an absolute turnaround time of 60 minutes. The study was conducted under 150 adult patients with suspected urinary tract infections, of whom 61.3% were female and 38.7% were male. *E. coli* was the predominant uropathogen (55.3%), followed by *K. pneumoniae* (19.3%).

The principal contribution of the present study is not only the discovery of *bla*_{CTX-M-15} and *aac*(6')-Ib-cr as novel resistance targets but rather, the simple and cost effectiveness methodological and molecular workflow that can be conducted in low resource laboratory at point of care to detect the specific antimicrobial resistance gene of *bla*_{CTX-M-15} and *aac*(6')-Ib-cr using a direct urine sample processing which undergoes DNA extraction in which the targeted antimicrobial genes detected by the PCR by the principle of amplification. This is important because the practical value of a molecular diagnostic workflow method depends not only on the resistance determinant being detected but also on the cost effectiveness, turnaround time, and the friendly use by laboratory scientist detecting this specific antimicrobial resistance which is *bla*_{CTX-M-15} and *aac*(6').

The DNA extraction experimental procedures were demonstrated across the study population. A260/A280 ratios ranged from 1.62 to 1.89, with a mean of 1.76 ± 0.08 and median of 1.77. Basically, the all the 150 samples were within the DNA purity normal range of 1.6–2.0.

The PCR statical detection of the of the antimicrobial gene of *aac*(6')-Ib-cr assay resulted the percentages of 92.63% sensitivity and 92.73% specificity, with a PPV of 95.65%, NPV of 87.93%, and overall accuracy of 92.67%. The corresponding exact 95% confidence intervals were 85.41–96.99% for sensitivity and 82.41–97.98% for specificity. The classification included 88 TP, 51 TN, 4 FP and 7 FN results. These findings indicate that the assay was able to identify most samples classified as resistant by the reference method while maintaining a relatively low number of false-positive results.

The agreement between the *aac*(6')-Ib-cr molecular assay and AST was further supported by a Cohen's κ of 0.844, indicating high agreement. The statistically significant Pearson association ($\chi^2 = 107.02$, $df = 1$, $P < 0.001$) provides additional evidence of a strong relationship between the molecular and reference classifications. Furthermore, McNemar's test was not statistically significant ($\chi^2 = 0.36$, $P = 0.546$), suggesting that there was no evidence of systematic directional disagreement between the paired methods. The ROC analysis yielded an AUC of 0.927, further supporting strong discriminatory performance.

The *bla*_{CTX-M-15} antimicrobial resistance gene also produced a statical performance with the percentages of sensitivity resulting 94.74%, specificity was 90.91%, PPV was 94.74%, NPV was 90.91%, and overall accuracy was 93.33%. The exact 95% confidence intervals were (88.14 to 98.27%) for sensitivity and (80.05% to 96.98%) for specificity. These findings indicate that the assay detected some of samples classified as resistant by AST while also correctly excluding most susceptible samples [13].

Agreement for *bla*_{CTX-M-15} was also high, with a Cohen's κ of 0.856. The association between molecular and phenotypic results was statistically significant (Pearson's $\chi^2 = 110.03$, $df = 1$, $P < 0.001$), while McNemar's test did not identify statistically significant systematic disagreement ($\chi^2 = 0.10$, $P = 0.752$). The ROC AUC of 0.928 indicates strong interval between the reference-positive and reference-negative threshold.

The four false-positive and seven false-negative results for *aac*(6')-Ib-cr, together with the five false-positive and five false-negative results for *bla*_{CTX-M-15} which demonstrate that the molecular and phenotypic approaches are not completely interchangeable. Genotypic detection shows the presence of the specific resistance gene, whereas AST measures the phenotypic response of the organism to antimicrobial agents [14,15,16]. Consequently, the presence of a resistance gene does not necessarily reproduce the complete phenotypic resistance profile, while a phenotypically resistant organism may possess a resistance mechanism not targeted by the present assay [17,18]. The discordance could reflect differences between genotype and phenotype, low target abundance, specimen-related inhibition, analytical variation, or limitations of the reference method [19,20].

The rapid turnaround time and the cost effectiveness are the most important practical characteristics of this molecular workflow. The complete process was reported to take approximately 60 minutes, compared with approximately 24–72 hours for conventional AST and approximately 24–48 hours for culture followed by AST [21]. This reduction in time could provide clinicians with early molecular information about selected resistance determinants while conventional microbiological testing is still in progress.

The cost-conscious nature of the workflow is another potential advantage. However, the current manuscript does not provide a sufficiently detailed numerical cost calculation to support a defensible exact cost-per-test claim. Irrespective of the various experimental procedure with a positive outcome, there were some outlined limitations, first we just used only 150 urine urinary tract infection samples for the entire experimental study of this research.

The Second limitation is that we only targeted on two antimicrobial resistance gene which are *bla*_{CTX-M-15} and *aac*(6').

The overall findings support the feasibility of a rapid direct-from-urine molecular workflow for early detection of selected antimicrobial resistance determinants. The strongest demonstrated characteristics are the 150-sample evaluation, 100% DNA extraction success, A260/A280 range of 1.62–1.89, approximately 60-minute turnaround, *aac*(6')-Ib-cr sensitivity of 92.63% and specificity of 92.73%, *bla*_{CTX-M-15} sensitivity of 94.74% and specificity of 90.91%, high agreement with AST ($\kappa = 0.844$ and 0.856), and AUC values of 0.927 and 0.928, respectively. The workflow was therefore positioned as an early molecular AMR detection or screening approach that complements rather than replaces culture and phenotypic AST

CONCLUSION

In conclusion, this molecular workflow study for the detection of *bla*_{CTX-M-15} and *aac*(6') antimicrobial resistance from midstream urine. Although some PCR-based methods already existed but relevantly this study mostly engaged in the detection of these two specific antimicrobial genes which *bla*_{CTX-M-15} and *aac*(6'), Simple rapid workflow, cost-effectiveness and to produce a turnaround result with 60minutes.

ACKNOWLEDGEMENT

The authors sincerely thank the management and staff of Parul Sevashram Hospital, Vadodara, Gujarat, India for providing us with the access to their modern laboratory facilities and for their support during sample collection and laboratory investigations. We are grateful to the Department of Medical Laboratory Sciences, Faculty of Allied and Healthcare Sciences, Parul University- Vadodara, India for providing the academic guidance, infrastructure, and the technical support necessary to conduct this research.

DECLARATION

Conflict of Interest: Authors declare no conflict of interest.

Funding Statement: No external funding was received for this study.

Ethical Approval Number: Approval No: PUIECHR/PIMSR/00/081734/8126

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